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# EXPRESSION OF CD40 BY THE CELLS OF BENIGN AND MALIGNANT BREAST TUMORS AND ANTITUMOR ACTION OF AUTOLOGOUS LYMPHOCYTES AGAINST CHEMORESISTANT AND CHEMOSENSITIVE TUMORS

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***Aim:*** **To study the expression of CD40 by cells of benign and malignant tumors of mammary gland, and to compare the efficacy of lymphocytes antitumor activity against drug resistant and sensitive breast tumors in relevance to CD40 expression.** ***Methods:*** **Breast tumor explants were cultured with autologous lymphocytes in double diffusion chambers. The results were evaluated by morphological criteria of explants growth. Expression level of molecules on tumor cells was analyzed using immunohistochemical method (paraffin embedded slides), and on lymphocytes — by the method of indirect immunofluorescence.** ***Results:*** **The highest level of CD40 expression was detected on cells of chemoresistant malignant breast tumors, and the lowest one — on cells of benign breast tumors. The decreased CD40 expression on lymphocytes from patients with drug resistant breast cancer was compared with that on lymphocytes of the patients with drug sensitive breast cancer. The study of antitumor activity of autologous lymphokine activated killer cells (LAK) has shown their pronounced antitumor activity against drug resistant malignant breast tumors.** ***Conclusion:*** **Marked antitumor activity of LAK from the patients with drug resistant breast cancer is associated with high expression level of CD40 on tumor cells and with its decreased expression on lymphocytes.**
***Key Words:*** **CD40, p53, CD54, cell proliferation, benign and malignant breast tumors, drug resistance, LAK.**

CD40 molecule is a 48 κDa protein that belongs to the superfamily of TNF receptors which contains a various number of cysteine rich domains as a characteristic pattern [1–3]. First, CD40 molecule was found on bladder cancer cells, and later — on normal and transformed B-lymphocytes [4, 5]. Also CD40 may be expressed by other antigen-presenting cells (dendritic cells, macrophages), endothelial, epithelial, and neural cells, keratinocytes, fibroblasts, $CD34^+$ hemopoietic precursor cells, as well by cells of tumors of different histogenesis and localization (tumors of mammary gland, intestine, stomach, nasopharynx, melanoma etc) [2, 6, 7]. CD40L, a 39 kD ligand of CD40, known as CD154 or gp39, is mainly expressed by T-lymphocytes [2, 8].

CD40 molecule plays a central role in immunoregulation and influences cell proliferation, activation and survival [9–11]. Interaction of CD40 and CD40L results in wide spectrum of effects on cells of immune system and on tumor cells. CD40 activation on tumor cells may alter tumor growth, in some cases leads to tumor growth inhibition, and in some cases — to growth stimulation or doesn't influence it at all [11–13].

Involvement of CD40 in antitumor defense may occur via involvement of different mechanisms: promotion of recognition by dendritic cells, induction of specific immunologic response with participation of B- and T-lymphocytes, stimulation of cytotoxic T-lymphocytes, natural killer cells, memory T-cells, production of various cytokines (GM-CSF, IL-1, IL-4, IL-6, IL-8, IL-10, IL-12, RANTES and TNFα), elevation of expression of costimulatory molecules on tumor cell surface, etc [14–17].

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*Abbreviations used:* IL-2 – interleukin-2; LAK – lymphokine activated killer cells; PBL – peripheral blood lymphocytes.

As has been shown in the studies of solid tumors and some lymphoproliferative diseases, CD40/CD40L interaction plays an important role in induction of apoptosis [12, 15].

Along with possibility of tumor cells death due to their interaction with T-lymphocytes expressing CD40L, such interaction may lead as well to accelerated tumor growth, which could be realized via numerous mechanisms: production of cytokines promoting tumor growth, in particular, secretion of angiogenic cytokines; promotion of adhesive properties etc. [13, 18].

The data of a number of authors have demonstrated that CD40 expression may be accompanied by the development of multiple drug resistance (to doxorubicine, vinblastin etc) by caspase-independent and caspase-dependent pathways [12, 19]. As we have shown earlier, the tumors resistant even to a single antitumor drug possess elevated sensitivity to the action of cytotoxic cells, in particular, when activated by IL-2, *in vitro* and *in vivo* [20].

The aim of the present study was to perform a comparative evaluation of the efficacy of antitumor action of lymphocytes against chemoresistant and chemosensitive breast tumors dependent on CD40 expression, and to analize the rate of CD40 expression by the cells of benign and malignant tumors of mammary gland. To find a possible relation between CD40 expression and antitumor action of lymphocytes, it was interestingly to study possible association between such action and the level of proliferative and adhesive activity of tumor cells and lymphocytes.

## MATERIALS AND METHODS

Tumor tissue samples and PBL were obtained from the patients with benign (n = 12; diagnosis — fibroadenoma, fibrocystoma mastopathy, fibroadenomatosis, macromastia) and malignant (n = 8; dia-

gnosis — breast carcinoma) breast tumors cured in the Department of Surgery of Kyiv Hospital № 1 (Kyiv, Ukraine). The studies were carried out in accordance to the International and State rules on Bioethics.

Tumor explants (the slices of tumor tissue < 0.2 $mm^3$ obtained during surgical treatment) were studied. Lymphocytes were isolated from whole heparinized blood by centrifugation in the ficoll-verografin density gradient. Individual sensitivity of tumor explants to antitumor drugs (doxorubicine (Ebeve, Austria) — 0.02 mg/ml, cyclophosphane (Olanpharm, Latvia), 5-fluorouracil (Ebeve, Austria) — 0.006 mg/ml, methotrexate (Teva Pharmaceutical Industries LTD, Israel) — 0.005 mg/ml) was determined by cultivation of explants in diffusion chambers in culture medium supplemented with mentioned drugs.

To produce lymphokine activated killer cells (LAK), lymphocytes were incubated with RIL-2 (1000 MU/ml) (BIOTECH, Russia) for 2 h at 37 °C in 5% $CO_2$, and twice washed.

Antitumor activity of lymphocytes has been analyzed by the patterns of tumor explants growth upon their co-cultivation in diffusion chambers. Tumor cells and lymphocytes were co-cultivated for 5 days in complete RPMI-1640 medium (Sigma, USA) at 37 °C in atmosphere of 5% $CO_2$. Then the filters of diffusion chambers were fixed, stained by Karacchi hematoxylin, treated by spirits (50°, 70°, 96°, 100°) and xylene, and preparations for microscopic examination were prepared using canadian balsam.

The evaluation of PBL and LAK antitumor activity was done based on morphological patterns of explant's growth: destruction of tumor cells, the absence of tumor cell migration from explant, migration of single tumor cells from explant, formation of monolayer of different density; formation of cell conglomerates; formation of spheroids [20].

Expression of CD40, p53 and antigen of proliferating cells IPO-38 by tumor tissue samples or PBL of the patients was determined with the use of respective monoclonal antibodies (mAbs) (IEPOR NASU). Anti-CD40 mAb were kindly provided by Dr. Edward A.Clark (University of Washington, Department of Immunology, Seattle, USA). mAbs were used at the concentration of 40 μg/ml. To determine the expression of mentioned proteins on tumor tissue, surgically resected tissue samples were fixed in formalin, and after standard histological treatment were placed in paraffin blocks. For immunohistochemical study, the 4–5 μm slides were treated with the respective mAbs and secondary complex EnVision (DAKO, Denmark). The level of protein expression was evaluated by semiquantative method (by the sum of scores for stained cells and by intensity staining) (Table 1) [21].

To determine the expression of CD40, CD54 and nuclear antigen of proliferating cells by PBL, the method of indirect immunofluorescence was used: the cells were stained by mentioned mAb, than incubated with secondary rabbit FITC-conjugated anti-mouse IgG antibodies (Sigma, USA). For detection of antigen of proliferating cells, lymphocytes were fixed for 5 min in 3.7% paraformaldeghyde solution (Sigma, USA), then treated with 0.2% Triton X-100 (Sigma, USA). After that the reaction was performed similarly to that for surface antigens. For the study, LUMAM-1 microscope was used. The percent of cells that bound fluorescence probe, was calculated.

**Table 1.** Semiquantative evaluation of immunohistochemical detection of the molecules (by Allred D.C.)

| 1. The part of positively stained cells | Score |
|---|---|
| 0 | 0 |
| 1–10 | 1 |
| 10–30 | 2 |
| 30–45 | 3 |
| 45–60 | 4 |
| 60–100 | 5 |
| 2. Staining intensity | |
| Negative | 0 |
| Low | 1 |
| Median | 2 |
| High | 3 |
| 3. Total score | |

*Note.* The level of proteins expression of was evaluated by the sum of scores for stained cells and by intensity of the staining [21].

Statistical analysis of the data was performed with the use of the methods of variation statistics.

## RESULTS AND DISCUSSION

In our study of the expression of CD40, CD54 and nuclear antigen of proliferating cells IPO-38 by drug resistant (n = 6) and drug sensitive (n = 2) malignant and benign breast tumors it was shown that the highest expression level of CD40 was present on chemoresistant tumor cells, while the lowest one — on the cells of benign tumors. It has been also recorded that there is an elevation of IPO-38 and p53 expression in the cells of drug resistant tumors compared with drug sensitive ones. Their lowest expression level was observed in the cells of benign tumors (Tables 2 and 3, Figs. 1, 2).

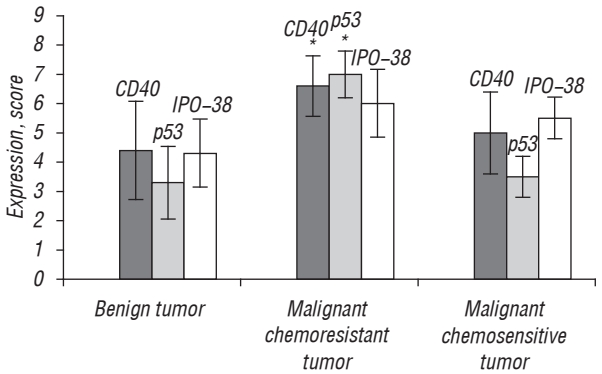


**Fig. 1.** Expression of CD40, p53 and antigen of proliferating cells by benign and malignant breast tumor cells resistant or sensitive to antitumor drugs. *Reliable difference between p53 and CD40 expression by tumor cells of malignant and benign tumors ($P < 0.5$).

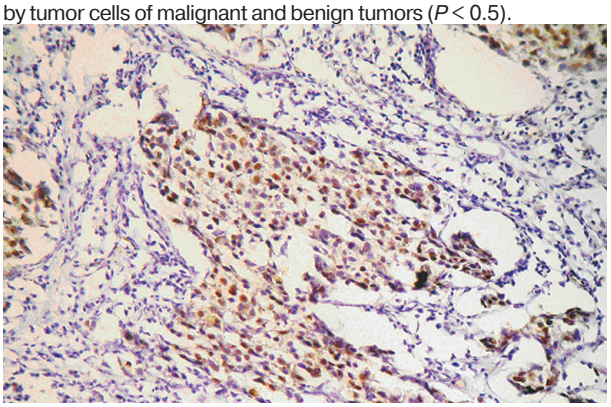

**Fig. 2.** Expression of CD40 by drug resistant breast cancer tumor cells, × 200

**Table 2.** Expression of CD40, p53 and antigen of proliferating cells (IPO-38) by drug resistant breast carcinoma cells (immunohistochemical scores)

| Case number | Diagnosis | Sensitivity to the action of drugs | Expression, score | | |
|---|---|---|---|---|---|
| | | | CD40 | IPO-38 | p53 |
| 1 | Resistant carcinoma | Doxorubicine Metotrexate | 8 | 7 | 8 |
| 2 | Resistant carcinoma | Cyclophosphane Metotrexate 5-fluorouracil | 7 | 6 | 8 |
| 3 | Resistant carcinoma | Doxorubicine Cyclophosphane Methotrexate | 7 | 3 | 7 |
| 4 | Resistant carcinoma | Doxorubicine Metotrexate | 6 | 7 | 7 |
| 5 | Resistant carcinoma | Cyclophosphane Metotrexate 5-fluorouracil | 5 | 7 | 6 |
| 6 | Resistant carcinoma | Doxorubicine Metotrexate 5-fluorouracil | 7 | 6 | 7 |

*Note.* Score – semiquantitive evaluation by Allred D.C.

**Table 3.** Expression of CD40, p53 and antigen of proliferating cells (IPO-38) by cells of benign breast tumors (immunohistochemical scores)

| Case number | Diagnosis | Expression, score | | |
|---|---|---|---|---|
| | | CD40 | IPO-38 | p53 |
| 1 | Fibroadenoma | 6 | 6 | 5 |
| 2 | Fibroadenoma | 5 | 3 | 3 |
| 3 | Fibroadenoma | 6 | 5 | 3 |
| 4 | Phyloid fibroadenoma | 7 | 5 | 2 |
| 5 | Fibroadenoma | 5 | 6 | 4 |
| 6 | Fibroadenoma | 6 | 4 | 4 |
| 7 | Fibroadenoma | 4 | 3 | 3 |
| 8 | Focalfibroadenomatosis | 4 | 5 | 0 |
| 9 | Cyclomastopathy | 2 | 3 | 3 |
| 10 | Cyclomastopathy | 3 | 3 | 3 |
| 11 | Papilloma with ulcerations | 2 | 4 | 2 |
| 12 | Macromastia | 3 | 5 | 3 |

*Note.* Score – semiquantitive evaluation by Allred D.C.

The level of CD40, CD54 and antigen of proliferating cells was studied in parallel on PBLs from breast cancer patients. We have shown that CD40 and IPO-38 expression by PBLs from the patients with drug resistant breast cancer was decreased, CD54 expression was elevated compared with these indexes in the case of drug sensitive breast tumors. The levels of CD40 and IPO-38 expression by PBLs of patients with benign tumors were significantly higher than those in patients with malignant tumors (Tables 4, 5).

The study of antitumor activity of non-activated and IL-2-activated PBLs showed that LAK from the patients with drug resistant breast cancer were active in the majority of cases: such activity manifested itself by an absence of tumor cell migration from explants or migration of single cells. In the control, only migration of cells from explant and formation of monolayer of low or medium density was observed. Non-activated lymphocytes from patients with drug resistant tumors in the majority of cases possessed weak antitumor activity (Table 6).

**Table 4.** Expression of CD40, CD54 and antigen of proliferating cells (IPO-38) by PBLs from patients with drug resistant malignant breast tumors (immunofluorescence)

| Case number | Diagnosis | Expression, % | | |
|---|---|---|---|---|
| | | CD40 | IPO-38 | CD54 |
| 1 | Resistant carcinoma | 4 | 5 | 18 |
| 2 | Resistant carcinoma | 10 | 3 | 10 |
| 3 | Resistant carcinoma | 6 | 5 | 8 |
| 4 | Resistant carcinoma | 5 | 6 | 15 |
| 5 | Resistant carcinoma | 5 | 7 | 8 |
| 6 | Resistant carcinoma | 6 | 3 | 8 |

*Note.* % – percent of positive cells.

**Table 5.** Expression of CD40, CD54 and antigen of proliferating cells (IPO-38) by PBLs from the patients with benign breast tumors (immunofluorescence)

| Case number | Diagnosis | Expression, % | | |
|---|---|---|---|---|
| | | CD40 | IPO-38 | CD54 |
| 1 | Fibroadenoma | 6 | 15 | 14 |
| 2 | Fibroadenoma | 11 | 9 | 10 |
| 3 | Fibroadenoma | 10 | 15 | 4 |
| 4 | Phyloid fibroadenoma | 12 | 10 | 5 |
| 5 | Fibroadenoma | 10 | 6 | 10 |
| 6 | Fibroadenoma | 10 | 5 | 10 |
| 7 | Fibroadenoma | 5 | 5 | 8 |
| 8 | Focalfibroadenomatosis | 5 | 5 | 15 |
| 9 | Cyclomastopathy | 12 | 8 | 14 |
| 10 | Cyclomastopathy | 10 | 5 | 5 |
| 11 | Papilloma with ulcerations | 10 | 5 | 10 |
| 12 | Macromastia | 4 | 7 | 10 |

*Note.* % – percent of positive cells.

In contrary, non-activated and activated by IL-2 PBLs from the patients with benign tumors in the majority of cases did not possess antitumor activity: tumor growth pattern practically did not differ from the control.

Drug resistant tumor cells demonstrated elevated sensitivity to the LAK action. The obtained results showed that the pronounced antitumor activity of LAK was associated with high expression level of CD40, p53 and antigen of proliferating cells (IPO-38) by tumor cells, while on patients' PBLs decreased expression of CD40 and IPO-38 and increased CD54 expression were detected.

So, we addressed the questions: what mechanisms caused such elevated sensitivity of drug resistant tumors to the applied adoptive immunotherapy approach, and what are the possible ways of CD40 impact on antitumor activity of lymphocytes. Unfortunately, the respective data are scarce, but according to the literature data, IL-2-activated lymphocytes acquire some properties favoring active lysis of target cells: elevation of adhesion molecules expression, promotion of lymphocytes interaction with tumor cells, synthesis and secretion of different cytokines by LAK, etc [22–25]. We demonstrated that the highest expression level of CD54 (ICAM-1) was observed on lymphocytes from the patients with drug resistant breast cancer. It could be suggested that among the factors influencing elevated sensitivity of LAK to drug resistant tumors, high expression level of adhesion molecules could be important. During the development of drug resistance,

**Table 6.** Sensitivity of breast carcinoma explants to antitumor activity of PBL and LAK (morphological patterns of explants growth)

| Case number | Diagnosis | Antitumor activity of lymphocytes | | |
|---|---|---|---|---|
| | | Growth of explantats (control) | Action of PBL | Action of LAK |
| 1 | Resistant carcinoma | Monolayer of medium density | Monolayer of low density | No migration |
| 2 | Resistant carcinoma | Monolayer of medium density | Monolayer of low density | Monolayer of low density |
| 3 | Resistant carcinoma | Migration of single cells | Migration of single cells | No migration; destruction |
| 4 | Resistant carcinoma | Monolayer of medium density | No migration | No migration |
| 5 | Resistant carcinoma | Monolayer of medium density | Monolayer of medium density | Initial stage of monolayer formation |
| 6 | Resistant carcinoma | Monolayer of low density | Initial stage of monolayer formation | Migration of single cells |
| 7 | Fibroadenoma with microcalcification (carcinoma *in situ*); chemosensitive | Migration of single cells | Migration of single cells | No migration |
| 8 | Chemosensitive carcinoma | Monolayer of medium density | No migration | No migration; destruction |

elevated sensitivity to the LAK action may be also caused by such factors as altered expression of some membrane proteins on tumor cells, for example, P-gp, production of ATP by tumor cells (promoting LAK cytotoxicity, changing tumor cell adhesive properties, etc) [26–28].

Induction of apoptosis in different types of tumor cells is one of the main mechanisms of CD40 inhibiting influence on tumor growth [5, 29]. This statement is supported by the fact that upon CD40 activation, expression of FasL, TRAIL (Apo-2L), Fas and ICAM-1 on tumor cell surface and expression of cytokines IL-1, IL-6, IL-8, IL-10, IL-12, GM-CSF, TNFα is observed [17, 30]. Despite the fact that the death domain is absent in C-terminus of CD40 molecule, the ability of this molecule to transfer death signals to the nucleus is realized via adapter proteins of TRAF family (TNF Receptor-Associated Factor), TRAF2 and TRAF6 [29, 31].

The basic mechanism of tumor growth suppression upon CD40 up-regulation is stimulation of immune system cells, involved in antitumor defense. In particular, CD40 promotes antigen-presenting functions of dendritic cells, macrophages, monocytes, and production of cytokines (IFNγ, IL-12). This increases cytotoxicity of macrophages and dendritic cells, and induces expression of antiapoptotic Bcl-2 protein by dendritic cells, as well as antibody-dependent cytotoxicity of natural killer cells, cytotoxic T-lymphocytes and memory T-cells [12, 17, 32].

Expression of CD40 by B-lymphocytes favors their enhanced proliferation, differentiation, expression of co-stimulatory molecules and antigen presentation [33]. As a result of B lymphocytes activation, induction of antitumor T-cell response occurs due to the direct influence on B lymphocytes and indirect influence on other antigen presenting cells [12].

In conclusion, we suggest that the increased sensitivity of drug resistant breast tumor cells expressing CD40 to the LAK action may be mediated by expression of adhesion molecules in parallel with activation of cytotoxic cells, and possibly — by apoptotic mechanisms. However, this problem requires further studies. The conclusions are grounded on uniformity of the data obtained in the study of samples of drug resistant tumors and their comparison with large control group of the patients with benign tumors (n = 12).

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# ЭКСПРЕССИЯ CD40 КЛЕТКАМИ ДОБРОКАЧЕСТВЕННЫХ И ЗЛОКАЧЕСТВЕННЫХ ОПУХОЛЕЙ МОЛОЧНОЙ ЖЕЛЕЗЫ И ПРОТИВООПУХОЛЕВОЕ ДЕЙСТВИЕ LAK В ОТНОШЕНИИ ХИМИОРЕЗИСТЕНТНЫХ И ЧУВСТВИТЕЛЬНЫХ ОПУХОЛЕЙ

***Цель:*** **изучение частоты экспрессии CD40 клетками злокачественных и доброкачественных опухолей молочной железы и сравнение эффективности противоопухолевого действия лимфоцитов в зависимости от экспрессии CD40 в отношении резистентных и чувствительных опухолей молочной железы.** ***Методы:*** **культивирование эксплантатов опухолей молочной железы с аутологичными лимфоцитами в двойных диффузионных камерах. Оценку результатов проводили на основании морфологических критериев роста эксплантатов. Для определения экспрессии молекул на опухолевых клетках использовался иммуногистохимический метод (парафиновые срезы), а на лимфоцитах — метод непрямой иммунофлуоресценции.** ***Результаты:*** **наиболее высокий уровень экспрессии молекул CD40 отмечен на клетках резистентных злокачественных опухолей молочной железы по сравнению с опухолями, чувствительными к химиопрепаратам, а наиболее низкий — на клетках доброкачественных опухолей. Установлено снижение экспрессии CD40 лимфоцитами больных со злокачественными резистентными опухолями молочной железы по сравнению с лимфоцитами больных с чувствительными опухолями. На лимфоцитах больных с доброкачественными опухолями уровень экспрессии CD40 был значительно выше по сравнению со злокачественными. Изучение противоопухолевой активности аутологичных ЛАК показало, что противоопухолевое действие у больных со злокачественными опухолями, резистентными к химиопрепаратам, было более выражено.** ***Выводы:*** **выраженная противоопухолевая активность ЛАК больных со злокачественными опухолями, резистентными к химиопрепаратам, ассоциируется с высоким уровнем экспрессии CD40 на опухолевых клетках и со снижением его экспрессии на лимфоцитах.**

***Ключевые слова:*** **CD40, p53, CD54, пролиферация клеток, доброкачественные и злокачественные опухоли молочной железы, химиорезистентность, ЛАК.**